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Bacterial biofilm formation on indwelling urethral catheters

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Abstract

Urethral catheters are the most commonly deployed medical devices and used to manage a wide range of conditions in both hospital and community care settings. The use of long-term catheterisation, where the catheter remains in place for a period >28 days remains common, and the care of these patients is often undermined by the acquisition of infections and formation of biofilms on catheter surfaces. Particular problems arise from colonisation with urease-producing species such as *Proteus mirabilis*, which form unusual crystalline biofilms that encrust catheter surfaces and block urine flow. Encrustation and blockage often lead to a range of serious clinical complications and emergency hospital referrals in long-term catheterised patients. Here we review current understanding of bacterial biofilm formation on urethral catheters, with a focus on crystalline biofilm formation by *P. mirabilis,* as well as approaches that may be used to control biofilm formation on these devices.

Keywords: Biofilms, Disease processes, Diseases, Resistance, Virulence

Introduction

Urethral catheters have been used in human medicine for over 3500 years. The term catheter derives from the ancient Greek *kathiénai*, which can be translated as “to thrust into” or “to send down”, and describes a medical device used to drain fluid from a body cavity (Mattelaer and Billiet 1995; Feneley *et al*. 2015). The Foley catheter, widely used in medical practice today, first became commercially available in the late 1930s, and the basic design of these devices remains largely unchanged in modern versions (Foley 1937; Feneley *et al.* 2015). These devices are most commonly made from latex or silicone, and consist of a flexible tube inserted through the urethra into the bladder, where it is held in place by a retention balloon inflated with a saline solution. There are two channels running through the

tube; one to allow the inflation of the retention balloon, and the other to allow the drainage of urine from the bladder *via* an eye-hole in the catheter tip (**Figure 1**).

These devices are deployed to treat numerous conditions related to bladder dysfunction and management of urine output, with duration of catheterisation ranging from short-term usage (up to ~7 days) in clinical settings such as hospitals, to long-term use (28 days and longer) in patients cared for in the community or in nursing home settings (Stickler 2008; Loveday *et al.* 2014; Stickler 2014; Shackley *et al.* 2017). The latter includes the use of long-term urethral catheterisation to manage urinary incontinence in elderly individuals and those with spinal cord injuries, as well as to minimise pressure ulcers and skin breakdown in immobile patients (Stickler 2008; Stickler 2014; Feneley *et al.* 2015).

Urinary catheters are now the most commonly used medical devices worldwide, with estimates of over 100 million urethral catheters sold annually (Saint *et al.*, 2000), and over 30 million urinary catheters fitted every year in the USA alone (Darouiche 2001). Although these simple devices can provide considerable benefit to many individuals, their use undermines natural defences of the urinary tract (see below). Thus management of catheterised patients is frequently complicated by infection where formation of biofilms is a key feature (Kunin *et al.* 1997; Warren 2001; Stickler 2008; Stickler 2014). Given the prolific use of urinary catheters in modern medicine, it is therefore unsurprising that catheter-associated urinary tract infections (CAUTI) are among the most common nosocomial infections (Loveday *et al.* 2014; Feneley *et al.* 2015). The prevalence of CAUTI is also associated with a significant financial and human cost, estimated to be £1.0-2.5 billion and up to 2100 deaths per year within the UK's National Health Service (NHS) (Feneley *et al.* 2015).

Catheter-associated urinary tract infection

The introduction of a catheter circumvents innate barriers to microbial colonisation in the urinary tract, such as the continual sloughing of urethral epithelial cells and innate mucosal immune function, as well as the periodic flushing action of urine expelled from the bladder which efficiently prevents attachment and rebuffs invading microbes (Warren 2001; Jacobsen et al. 2008; Stickler 2014; Feneley *et al.* 2015). In contrast, urethral catheterisation presents bacteria with a readily-colonisable abiotic surface and promotes a slow continuous flow of urine from the bladder, effectively providing a "bridge" between the nutrient rich bladder and external environment (Warren 1991; Warren 2001; Stickler 2014).

Bacteria may migrate from the skin surrounding the urethral opening into the urinary tract, using the external surfaces of the catheter, or in some cases may be introduced directly into the bladder on the catheter itself, if aseptic handling practices are not observed when fitting catheters (Warren 2001; Nicolle 2005; Stickler 2014). If the closed drainage system of the catheter is breached (for example during emptying or changing of drainage bags), bacteria can contaminate the system and ascend intra-luminally through the catheter into the bladder (Warren 2001; Nicolle 2005; Stickler 2014). Additionally, the design of the catheter and placement of the inflation balloon results in the formation of a residual pool of urine in the bladder, which is continually replenished with fresh nutrients from the kidneys (Warren 2001; Nicolle 2005; Stickler 2014). This creates an ideal environment for dense bacterial growth, and, once organisms have taken advantage of the catheter to reach this location, they are provided with ideal conditions in which to flourish.

Although the majority of catheters deployed in the NHS will be used for short-term catheterisation in hospitalised patients, a notable proportion will be used for long-term indwelling catheterisation of patients in community care or nursing home settings (Royal

College of Physicians 2010; Prinjha and Chapple 2013; Shackley *et al*. 2017). A recent prospective study of urinary catheter use in over 9 million NHS patients indicated that around 22.2% of catheterised patients undergo long-term indwelling catheterisation (equating to >256,000 patients over the ~4 year study period), with the majority of these patients cared for in the community (>180,000 patients) (Shackley *et al.* 2017). Furthermore, national audits suggest that for patients aged over 65, ~7% cared for in the community and ~10% of nursing home residents may be living with permanent urethral catheterisation (Royal College of Physicians 2010).

Since the risk of CAUTI increases with duration of catheterisation, and patients in community care are usually not subject to the continuous clinical monitoring intrinsic to hospital care, this group of individuals are not only at high risk of infection but also more likely to be harmed by the complications that can arise as a consequence of CAUTI and associated biofilm formation (Kohler-Ockmore and Fenely 1996; Stickler 2014). Congruent with this are estimates suggesting the cost of treating CAUTI and associated complications in long-term catheterised patients in the community may be as high as £10,000 per patient, along with studies highlighting the prevalence of emergency hospital referrals in this group (Kohler-Ockmore and Fenely 1996; Evans *et al.* 2000). The use of these devices and the size of the global urinary catheter market is predicted to continue to grow, along with the complications associated with the use of current catheter designs and the formation of bacterial biofilms on these devices (Prinjha and Chapple 2013; Feneley *et al.* 2015).

Biofilm formation and catheter encrustation

A biofilm can be defined as a surface-associated microbial community comprised of cells embedded within a matrix of extracellular polymeric substances (EPS) (Donlan 2002; Donlan and Costerton 2002; Fux *et al.* 2005). The majority of bacterial cells in nature are

believed to exist in biofilm communities, which provides benefits in dealing with environmental stresses and confers a survival advantage over growth in the planktonic state (Donlan 2002; Donlan and Costerton 2002; Fux *et al.* 2005). Biofilm-associated cells exhibit reduced growth rates, distinct physiological characteristics, and altered gene expression compared to their planktonic counterparts (Donlan and Costerton 2002; Hall-Stoodley *et al.* 2004; Fux *et al.* 2005). The EPS surrounding the community also protects cells from harmful agents, by acting as a diffusion barrier or neutralizing or binding the agent, as well as providing mechanical support and resistance to shear stresses generated by flow of the surrounding milieu (Donlan and Costerton 2002; Fux *et al.* 2005; Flemming *et al.* 2007). These characteristics mean that biofilms which develop in clinical settings and on implanted medical devices often present a particular challenge in terms of infection control and treatment (Donlan 2001). The structure and physiological characteristics of biofilms confer protection from normal immune clearance and resistance to antimicrobials, even if constituent microbes are fully susceptible in planktonic culture (Donlan 2001; Hall-Stoodley *et al.* 2004; Fux *et al.* 2005; Touzel *et al.* 2016).

Biofilms form readily on urinary catheters, aided by the characteristics and topology of the catheter surface, the formation of conditioning layers, and the constant supply of nutrients from urine flowing through them (Stickler *et al.* 1993; Stickler *et al.* 1998; Downer *et al*. 2003; Stickler 2008). In particular, it has been demonstrated that irregularities and surface striations around the catheter eye-hole derived from the manufacturing process facilitate the initial adhesion of bacterial cells to the catheter (Stickler *et al.* 2003; Stickler 2008). Latex catheters may also contain embedded diatom skeletons that act as sites for bacterial attachment, and result from the use of diatomaceous earth in the injection moulding process (Stickler *et al.* 2003; Stickler 2008; Stickler and Morgan 2008).

Following the insertion of a urinary catheter, a conditioning film derived from constituents of the urine and host proteins such as fibrinogen can form on the catheter surface, which also supports bacterial adhesion and the initiation of biofilm formation (Donlan and Costerton 2002; Stickler 2008; Stickler and Morgan 2008). Because patients catheterised for a period of 4 weeks or more are almost certain to become bacteriuric, catheters in many individuals undergoing long-term catheterisation will be exposed to contaminated urine for a considerable period of time. This also means that newly inserted catheters may be quickly colonised and biofilms rapidly established (Warren 2001; Stickler 2008; Stickler 2014).

While *Escherichia coli* remains the most commonly isolated bacterial pathogen in uncomplicated UTI, a broader range of species are prevalent in CAUTI including *Proteus mirabilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa, Morganella morganii, Providencia* sp. and *Klebsiella pneumoniae* (Warren 2001; Macleod and Stickler 2007; Stickler 2008). *P. mirabilis, Ps. aeruginosa, E. coli,* and *E. faecalis* are the most commonly isolated pathogens, and either single species or polymicrobial biofilms may form on catheters, although in patients with long-term catheters, polymicrobial communities are more likely (Warren 2001; Macleod and Stickler 2007; Stickler 2008).

As modelling mixed species biofilms is more challenging than single species biofilms, there are fewer studies focused on polymicrobial CAUTI and biofilm formation, and further research is required in this area (Norsworthy and Pearson 2017). However, studies utilising a representative *in vitro* model of the catheterised urinary tract have proven useful in studying the formation of mixed species biofilms on urinary catheters (Stickler *et al.* 1999; Macleod and Stickler 2007). Importantly, these studies have highlighted the potential for antagonistic interactions between species that commonly infect the catheterised urinary tract (Macleod and Stickler 2007). Of note was the apparent exclusion of the particularly

problematic CAUTI pathogen *P. mirabilis,* which causes catheter encrustation and blockage through formation of unusual crystalline biofilms, by species such as *Enterobacteria cloacae* and *Ps. aeruginosa* (Macleod and Stickler 2007).

Crystalline biofilm formation is the cause of many of the most important and common clinical complications that are associated with long-term urethral catheterisation (Kohler-Ockmore and Feneley 1996; Kunin *et al.* 1997; Jacobsen et al. 2008; Stickler 2008; Stickler 2014; Feneley *et al.* 2015). This is compounded by the potential for encrustation and blockage to occur rapidly without warning, and the majority of long-term catheterised patients being managed in community care settings (Kohler-Ockmore and Feneley 1996; Stickler *et al*. 2006a; Long *et al.* 2014; Stickler 2014). Together, these factors mean blockage is often not noticed until more serious complications develop (Kohler-Ockmore and Feneley 1996; Sticker *et al.* 2006a; Long *et al.* 2014; Stickler 2014).

Catheter blockage can result in urine leakage causing incontinence, but often leads to accumulation of infected urine in the bladder, and eventual reflux of infected urine to the upper urinary tract and kidneys. Reflux of infected urine can subsequently initiate serious sequelae such as pyelonephritis, septicaemia, and shock (Warren 2001; Jacobsen *et al.* 2008; Stickler 2014; Feneley et al. 2015). Additionally, because the crystalline deposits are hard and abrasive, encrustations that form on the catheter tip and balloon can cause trauma to the bladder mucosa and urethra on catheter removal (Stickler 2008). Deflation of the catheter balloon can lead to fragmentation of encrustations which remain in the bladder where they further damage and irritate the bladder mucosa, and act as foci for the formation of bladder stones and reinfection of the catheterised urinary tract (Stickler 2008; Feneley *et al.* 2015). The crystalline nature of these biofilms also further contributes to the recalcitrance of these communities to antibiotics, with mineralisation of the biofilm shown to provide

greater protection of bacterial cells from antimicrobials compared to non-crystalline biofilms (Li *et al.* 2016).

It has been suggested that ~50% of patients undergoing long-term catheterisation will experience catheter blockage, and in many cases this will become a recurrent long-term problem (Kohler-Ockmore and Feneley 1996; Kunin *et al.* 1997; Mathur *et al.* 2005). It is therefore unsurprising that catheter blockage results in many emergency referrals which place a strain on healthcare systems. The scale of this problem was highlighted by Kohler-Ockmore and Feneley (1996) who recorded 506 emergency referrals from a group of 457 long-term catheterised patients over a 6 month period, mainly as a result of catheter blockage (Kohler-Ockmore and Feneley 1996).

Proteus mirabilis and crystalline biofilm formation

The formation of crystalline biofilms is the result of infection with urease producing bacterial species including *Providencia rettgeri, P. vulgaris,* and *P. mirabilis,* with the latter being the main cause of catheter encrustation and blockage (Stickler *et al.* 1993; Stickler *et al*. 1998, Macleod and Stickler 2007; Broomfield et al. 2009). *P. mirabilis* is not usually an early coloniser of the catheterised urinary tract, but with increasing duration of catheterisation the likelihood of *P. mirabilis* colonisation increases, and this species can be isolated from up to 40% of long-term catheterised patients experiencing catheter encrustation and blockage (Macleod and Stickler 2007). The combination of a strong ability to form biofilms on catheter surfaces and production of a highly active urease are the key factors driving the encrustation of catheters by this species (**Figure 2**).

The ureolytic activity of *P. mirabilis* in the catheterised urinary tract leads to the generation of ammonia, and the elevation of urinary pH (Griffith *et al*. 1976; Jones and Mobley 1987; Stickler *et al.* 1993;). Under these alkaline conditions normally soluble constituents of the urine precipitate and begin to form crystals of magnesium ammonium phosphate (struvite) and calcium phosphate (hydroxyapatite) (Griffith *et al.* 1976; Hedelin *et al.* 1984; Cox and Hukins 1989; Stickler *et al.* 1993; Holling *et al.* 2014a). These crystals become trapped within the developing biofilms where their growth is stabilised and enhanced by the biofilm matrix, ultimately resulting in the formation of a mineralised biofilm structure (Stickler *et al.* 1993; Dumanski 1994; Stickler 2014; **Figure 2**). *P. mirabilis* urease has been shown to hydrolyse urea at a much greater rate and block catheters more rapidly than other urease producing bacteria associated with CAUTI (Jones and Mobley 1987; Broomfield *et al.* 2009). *P. mirabilis* catheter biofilms may also constitute highly alkaline microenvironments in otherwise acid or neutral urine, which could potentially facilitate localised crystallisation within the biofilm even when pH of the bulk urine is not optimal for crystal formation (Stickler *et al.* 1993a).

Using episcopic differential interference contrast (EDIC) microscopy, Wilks *et al*. (2015) have proposed that *P. mirabilis* crystalline biofilms form in four consecutive stages. These include the initial development of a foundation layer where small numbers of cells are initially supported by relatively large amounts of extracellular polysaccharide, followed by formation of a microcrystalline layer overlying this, before accumulation of larger amounts of crystalline material, and finally continued expansion of the biofilm to form the mature crystalline biofilm structure (Wilks *et al.* 2015). In contrast, other studies have indicated initial stages include the formation of a microcrystalline conditioning layer to which cells preferentially attach (Stickler and Morgan 2008).

Observations using Environmental Scanning Electron Microscopy (ESEM) of crystalline biofilms also provided further insight into the structure of these communities (Holling *et al.* 2014a). An important feature of ESEM is the capacity to image fully hydrated samples without the normal dehydration and fixation required for viewing samples by standard SEM. ESEM may also be coupled with techniques such as energy dispersive X-ray analysis (EDS) to investigate the chemical composition of structures observed (Bergmans *et al*. 2005; Hannig *et al*. 2010; Holling *et al*. 2014a). Application of ESEM and EDS to *P. mirabilis* crystalline biofilms has revealed the presence of delicate sheet-like crystalline structures of calcium phosphates, possibly derived from turnover of microcrystalline conditioning layers that form on catheter surfaces, as well as the extensive calcification of the biofilm matrix as a whole (Holling *et al.* 2014a).

Bladder stones that form in the residual pool of urine in the bladder are also common in long-term catheterised patients (Feneley *et al.* 2002; Sabbuba *et al.* 2004). These are also predominantly associated with *P. mirabilis* infection and believed to arise through an analogous process to crystalline biofilm formation (Bichler *et al.* 2002; Feneley *et al.* 2002; Sabbuba *et al.* 2004; Schaffer *et al.* 2016). The composition of bladder stones and crystalline biofilms is comparable, and the same strain of *P. mirabilis* may be found within the biofilm and bladder stones in a particular patient (Bichler *et al.* 2002; Sabbuba *et al.* 2004). *P. mirabilis* cells growing in the residual bladder urine or invading the bladder mucosa can act as foci for *de novo* stone formation, and viable cells have been found within these structures (Bichler *et al.* 2002; Sabbuba *et al.* 2004; Schaffer *et al.* 2016). Alternatively, encrustations that form on the exterior surfaces of the catheter and balloon while the catheter is in place, detach when the balloon is deflated during catheter removal, and initiate stone formation (Stickler 2008). The fact that *P. mirabilis* cells remain viable in stones is likely an important factor in the ability of this species to cause chronic infection and recurrent catheter blockage, with stones providing protection from mechanical, immune, and

antimicrobial clearing, as well as acting as reservoirs for reinfection following treatment or catheter changes (Sabbuba *et al.* 2004; Stickler 2014).

Mechanisms underlying *P. mirabilis* crystalline biofilm formation and virulence

In addition to urease production, *P. mirabilis* exhibits a number of other attributes and virulence factors that may be involved in crystalline biofilm formation and the pathogenesis of CAUTI. A particularly striking feature of *P. mirabilis* is its swarming motility, in which elongated, hyperflagellated swarmer cells form multicellular rafts and move rapidly over solid surfaces (Jones *et al.* 2004; Rather 2005; Morgenstein *et al.* 2010; **Figure 3**). This cyclic behaviour is responsible for the characteristic “bulls-eye” or terraced appearance of *P. mirabilis* colonies grown on agar plates, with each terrace representing one swarm cycle (**Figure 3**). Although swarming cells in *P. mirabilis* do not fully fit conventional definitions of a biofilm due to the motility of swarmer cell rafts, this highly coordinated multicellular behaviour shares many features with biofilm-associated cells. This includes altered gene expression, cell physiology, extracellular polysaccharide matrix production, and altered susceptibility to antimicrobials (Gygi *et al.* 1995; Jones *et al.* 2004; Rather 2005; Morgenstein *et al.* 2010).

Since *P. mirabilis* is capable of swarming over different types of catheter surfaces this motility has been implicated in its ability to migrate from the periurethral skin, along the catheter and into the bladder initiating CAUTI (Stickler and Hughes 1999; Sabbuba *et al.* 2002; Jones *et al.* 2004). Swarmer cells also exhibit an increased expression of virulence factors including urease, and it has been hypothesised that in conjunction with swarming motility this may facilitate expansion and spreading of crystalline biofilms across catheter surfaces (Stickler and Hughes 1999; Fraser *et al.* 2002; Sabbuba *et al.* 2002). Observations of *P. mirabilis* biofilms grown on catheters have also indicated the presence of swarmer cells within established biofilms (Stickler and Morgan 2006).

However, the role of swarming in pathogenesis of CAUTI and crystalline biofilm formation remains largely enigmatic. Neither swimming or swarming motilities have been found to be essential for crystalline biofilm formation and catheter blockage, with non-swarming mutants reported to have greater biofilm forming ability in some studies (Jones *et al.* 2005; Holling *et al.* 2104b). These observations also fit with transcriptomic analyses which have revealed a coordinated regulation of motile and adherent states in *P. mirabilis*, whereby flagellar biosynthesis and motility are repressed in response to signals that upregulate fimbrial production (Pearson and Mobley 2008; Pearson *et al*. 2010). Overall, present evidence suggests swarming may play a role in initial colonisation of the catheterised urinary tract, but that this motility is not essential for subsequent crystalline biofilm formation. However, there remains potential for differentiated swarmer cells to play a structural role in development of crystalline biofilms, unrelated to surface-associated motility.

The complete genome sequence of *P. mirabilis* has also shed light on mechanisms underlying the strong biofilm forming ability exhibited by *P. mirabilis* (Pearson *et al*. 2008b). Of particular relevance to biofilm formation are the large number of fimbrial operons encoded by *P. mirabilis*, affording the potential for generation of up to 17 distinct fimbrial structures in this species (Pearson *et al.* 2008b). Several *P. mirabilis* fimbria are already known to facilitate attachment to host cells and tissues as well as some catheter biomaterials, with conditioning layers that form on catheters thought to provide additional receptors for fimbriae and other adhesins (Bahrani, *et al*. 1994; Massad *et al*. 1994; Zunino *et al*. 2003; Jansen *et al.* 2004; Himpsl *et al.* 2008; Jacobsen *et al*. 2008; Stickler 2008; Pearson *et al.* 2008; Armbruster and Mobley 2012; Pellegrino *et al.* 2013; Scavone *et al*. 2016). However, although fimbriae-mediating host cell attachment have been shown to play a critical role in the pathogenesis of ascending UTI, further work is required to clearly elucidate which fimbria may be key to crystalline biofilm formation, and the role of many fimbria that may be produced by *P. mirabilis* remain unknown (Bahrani *et al.* 1994; Massad *et al.* 1994; Zunino

et al. 2003; Jansen *et al.* 2004; Himpsl *et al.* 2008; Pellegrino *et al.* 2013; Scavone *et al.* 2016).

Other functions related to crystalline biofilm formation specifically have been identified through construction and screening of mutants altered in ability to form biofilms. Holling *et al.* (2014b) used a random transposon mutagenesis approach to identify genes important to *P. mirabilis* biofilm formation and catheter blockage, which indicated an unexpected role for genes encoding efflux systems. Mutants disrupted in the putative multidrug *bcr/CflA* efflux pump were attenuated in ability to form crystalline biofilms and block catheters in an *in vitro* model of the catheterised urinary tract (Holling *et al.* 2014b). The role of efflux in biofilm formation in other pathogens has also been reported, with increased expression of efflux pumps noted in biofilm-associated cells compared to planktonic cells, the chemical inhibition or deletion of relevant genes resulting in attenuation or abolition of biofilm formation, and efflux linked with the increased antibiotic resistance of biofilms. (Kvist *et al.* 2008; Zhang and Mah 2008; Matsumara *et al.* 2011; Soto *et al.* 2013; Baugh *et al.* 2014; Holling *et al.* 2014b; Nzakizwanayo *et al.* 2017). Importantly, such studies also serve to highlight potential new targets for biofilm control, with subsequent investigations demonstrating that inhibitors of efflux in *P. mirabilis* can also reduce crystalline biofilm formation (Nzakizwanayo *et al*. 2017).

Although roles of motility, fimbria production, and efflux, have been studied in *P. mirabilis* biofilm formation, other mechanisms of multicellular coordination and biofilm formation well described in other pathogens are yet to be clearly elucidated in *P. mirabilis*. Key among these are quorum sensing (QS) systems that regulate population density dependant gene expression in a wide range of bacterial species, and contribute to the coordination of biofilm formation in other uropathogens such as *Ps. aeruginosa* (Chugani *et al.* 2001*;* Ng and Bassler 2009*;* Antunes *et al*. 2010). Although multicellular behaviours exhibited by *P.*

mirabilis (biofilm formation and swarming) are likely to involve considerable cell-cell communication, *P. mirabilis* lacks clearly identifiable homologues of the canonical LuxI synthase, and does not generate Acyl-homoserine lactones (AHL) utilised as QS signal molecules in other Gram negative species (Belas *et al.* 1998; Pearson *et al.* 2008). An intact AI-2 QS circuit (often involved in both inter and intra-species QS signalling) is also seemingly absent in *P. mirabilis*, and while production of the AI-2 signal molecule and a homologue to the AI-2 senor *luxS* has been identified, inactivation of *luxS* appears to have no significant impact on virulence and swarming (Schneider *et al.* 2002).

However, *P. mirabilis* appears to be able to still respond to exogenous AHLs generated by other species, which is reported to influence biofilm formation (Stanskowska *et al.* 2012). This has led to the hypothesis that *P. mirabilis* is able to modulate behaviours related to virulence in response to AHL production by other pathogens during polymicrobial infection (Armbruster and Mobley 2012). A range of other molecules have also been suggested to be detected and used by *P. mirabilis* to regulate virulence related gene expression in a QS-like fashion, including fatty acids, and putrescine, as well as diketopiperazines which may function as an alternative AHL-like signal molecule in *P. mirabilis* (Holden *et al.* 1999; Liaw *et al.* 2004; Sturgill and Rather 2004). However, the importance and role of these potential signalling molecules, and QS in general, to *P. mirabilis* biofilm formation and CAUTI remain unknown.

Approaches to control crystalline biofilm formation and catheter blockage

A wide range of strategies have been developed and evaluated in order to control CAUTI, and in particular the formation of crystalline biofilms and associated blockage of catheters. These range from those focused on the modification of catheter surfaces to prevent microbial adhesion or impart antimicrobial activities, to approaches seeking to offset the

formation of crystals through dietary modulation of urinary pH, and studies on behavioural interventions to catheter management in different healthcare settings (Jones *et al.* 2018). More recently, approaches based on new understanding of the molecular genetic basis of *P. mirabilis* crystalline biofilm formation, alternatives to conventional antimicrobial agents, and the use of early warning systems to signal the possibility of catheter blockage have been described (Stickler *et al.* 2003; Holling *et al.* 2014b; Nzakizwanayo *et al.* 2016; Milo *et al.* 2016; Milo *et al.* 2017; Nzakizwanayo *et al.* 2017; Milo *et al.* 2018).

Dietary intervention.

Dietary intervention represents a potentially simple, economical, widely applicable, and safe intervention, and several approaches have been evaluated for the control of encrustation specifically. In particular, the consumption of cranberry juice (or compounds derived from cranberry) has long been proposed to hold therapeutic or prophylactic potential in the control of uncomplicated UTI, and suggested to inhibit growth and adherence of uropathogens in the urinary tract (Bodel *et al.* 1959; Ofek *et al.* 1991; Avorn *et al.* 1994; Kuminski 1996; McMurdo *et al.* 2005; Thomas *et al.* 2017).

However, studies in which urine from volunteers consuming 1 L of cranberry juice per day was collected and used in *in vitro* models of *P. mirabilis* catheter infection, showed no significant impact on the production of crystalline biofilms and catheter blockage from consumption of cranberry juice specifically (Morris and Stickler 2001). Nevertheless, volunteers who consumed either cranberry juice or water as a control did generate urine that resulted in slower rates of biofilm formation in models, compared to urine from volunteers who had not consumed any additional fluids. Collectively these observations indicated increased fluid intake in general, but not cranberry juice specifically, is effective in reducing the rate of crystalline biofilm formation and prolonging catheter lifespan (Morris and Stickler

2001). Conversely, small scale clinical studies of concentrated proanthocyanins, extracted from cranberry, have indicated daily consumption in capsules could reduce the incidence of symptomatic CAUTI (Thomas *et al.* 2017). Although blockage specifically was not examined in this study, and it is not clear if this will also be effective in controlling encrustation.

The effects of increased intake of fluid on reducing encrustation rates are also in keeping with clinical observations relating to the variation in the threshold pH at which encrustation occurs in catheterised patients. This is termed the nucleation pH (pHn) and is the pH at which calcium and magnesium phosphates begin to precipitate from urine and crystal formation is initiated (Choong *et al.* 1999; Choong *et al.* 2001). Previous studies have indicated that catheterised individuals exhibiting recurrent blockage have significantly reduced pHn values compared to patients who were not exhibiting catheter blockage (Choong *et al.* 1999; Choong *et al.* 2001; Mathur *et al.* 2005). However, the pHn of urine varies both within a patient over time and between individuals, and it is possible to manipulate this factor in order to reduce the risk of encrustation and blockage through increased fluid intake (Choong *et al.* 1999; Choong *et al.* 2001; Suller *et al.* 2005; Mathur *et al.* 2005; Broomfield *et al.* 2009; Stickler and Morgan 2006).

Alternatively increasing the intake of dietary components that influence urinary pH may also be effective in reducing encrustation, and both clinical and laboratory studies have shown potential to do this through increased consumption of citrated drinks (Broomfield *et al*. 2009; Kahn *et al*. 2010). Notably, the intake of 1 L of citrate drink among catheterised patients significantly increased their average urinary pHn reducing the risk of catheter encrustation (Khan *et al.* 2010). However, while it is clear that increased fluid intake and consumption of citrated beverages is likely to be beneficial for catheterised patients, maintaining these kinds of dietary modifications over prolonged periods can be a challenge for many patients.

Surface modifications and antimicrobial catheters.

The formation of conditioning films and initial attachment of bacteria to catheter surfaces represents the first stages of biofilm formation, and preventing these events should provide effective control of catheter-associated biofilm formation. Strategies to inhibit these early stages of catheter colonisation involve modifying surface properties of catheters such as hydrophobicity, charge, and topology, or to incorporate antimicrobial agents intended to kill colonising microbes.

Catheters and other devices with hydrophilic surface coatings have been commercially available for some time, and intended to not only increase comfort of catheterised patients, but also to make these surfaces less vulnerable to the development of conditioning layers, and less attractive for bacterial colonisation (Donlan 2001; Stensballe *et al.* 2005). Examples of catheters in which hydrogel coatings have been augmented by incorporation of antimicrobial agents such as silver, or where catheter biomaterials have been directly impregnated with antimicrobial agents are also widely available (Morris *et al*. 1997; Maki *et al*. 2001; Pickard *et al*. 2012). Despite claims from some manufacturers that such devices are effective in preventing catheter encrustation, current evidence suggest these general strategies perform poorly in the control of *P. mirabilis* crystalline biofilm formation (Morris *et al.* 1997; Stickler and Morgan 2008; Desai *et al.* 2010; Pickard *et al.* 2012).

Studies evaluating the recalcitrance of numerous catheter types to crystalline biofilm formation, using *in vitro* models of the catheterised urinary tract, have demonstrated that all catheter types tested remained vulnerable to encrustation by *P. mirabilis* (Morris *et al.* 1997; Stickler *et al.* 2002; Stickler and Morgan 2008). These studies included those with hydrogel coatings, phosphorylcholine coatings, as well as silver containing hydrogels, or catheters impregnated with antibiotics such as nitrofurazone (Morris *et al.* 1997; Stickler *et al.* 2002;

Stickler and Morgan 2008). These laboratory studies are also supported by clinical observations, with recent large scale clinical trials of silver-alloy and nitrofurazone coated catheters demonstrating no significant impact on incidence of CAUTI in catheterised patients (Pickard *et al.* 2012). Although this trial focused on short-term catheterised patients in a hospital setting, it is most likely that such catheters would be even less effective in the control of colonisation in long-term catheterised individuals, where they must function effectively to exclude microbes for weeks or months at a time. This is congruent with direct observations of crystalline biofilm formation on hydrogel/silver coated catheters removed from patients, and the rapid formation of *P. mirabilis* induced encrustations on hydrogel/silver coated and nitrofurazone silicone catheters in laboratory models (Stickler and Morgan 2008).

In the case of antimicrobial agents, *P. mirabilis* is intrinsically resistant to a range of antibiotics and biocides, including nitrofurazone, and the continual elution of agents from catheters may also rapidly reduce levels of agents to concentrations that are no longer effective (Johnson *et al.* 1993a; Stickler and Morgan 2008; Stickler 2014). This depletion of antimicrobials is also believed to contribute to the development and selection of antimicrobial resistance (Stickler 2014; Feneley *et al.* 2015). Furthermore, the formation of microcrystalline conditioning layers may provide suitable attachment sites for cells whilst shielding them from antimicrobial agents, undermining the ability of hydrogel coatings and antimicrobial coatings to resist biofilm formation (Stickler and Morgan 2008). In the case of catheters containing antimicrobials, pioneering cells killed and attached to the surface may also contribute to the eventual failure of this control method, acting as foci for further attachment whilst providing protection to cells that adhere later. Nevertheless, incorporation of antimicrobial agents into catheters continues to be an area of active development. New approaches to incorporate antimicrobials into catheter biomaterials, along with the use of a combination of antimicrobial agents, have the potential to increase both longevity and

efficacy of these devices against challenging pathogens such as *P. mirabilis* (Fisher *at al*. 2015).

More recently, surface modification approaches based on generation of defined nanoscale surface patterns that are inhibitory to microbial growth and attachment have been described, and suggested to be of use in the control of catheter biofilms (Schumacher *et al*. 2008; Reddy *et al.* 2011; Mann *et al.* 2014; Vasudevan *et al.* 2014). For example the Sharklet™ micropatterned surface has been shown to inhibit the ability of uropathogenic *E. coli* and *S. aureus* to colonise and form biofilms in *in vitro* laboratory studies (Reddy *et al*. 2011; Mann *et al.* 2014). The nanoscale structures forming the topologies in surfaces such as the Sharklet™ are proposed to work by generating mechanical stress on colonising cells, forcing them to continually adjust contact angle relative to the surface structures, ultimately inhibiting attachment (Schumacher *et al*. 2008). However, it is currently unclear how well such surfaces will perform when tested against *P. mirabilis,* and under highly challenging conditions which promote crystalline biofilm formation. It would seem possible that formation of crystalline conditioning layers and encrustation by *P. mirabilis* could also overcome this approach as with other surface modification strategies.

Triclosan and Farco-fill.

The broad spectrum biocide triclosan has been widely used in numerous applications ranging from incorporation in many domestic products and surfaces, to use in dental hygiene products and surgical scrubs (Sticker *et al.* 2003). *P. mirabilis* has exquisite sensitivity to triclosan, and this agent has been shown to be effective in the control of crystalline biofilm formation and catheter blockage using *in vitro* bladder models inoculated with *P. mirabilis* alone, or in polymicrobial communities (Sticker *et al*. 2003; Jones *et al.* 2006; Stickler and Morgan 2008; Williams *et al*. 2008). Importantly, this agent may be effectively delivered to

the catheterised urinary tract over a sustained period of time by using concentrated solutions of triclosan to fill retention balloons of all silicone catheters (Stickler *et al*. 2003). Triclosan is able to easily diffuse through the silicone catheter material, into the surrounding urine and provide effective control of *P. mirabilis* and crystalline biofilm formation (Stickler *et al.* 2003).

The success of this approach has ultimately led to the development of a fully licensed product (Farco-fill®), available to patients on prescription. Although this approach undoubtedly benefits many individuals and has had considerable positive impact, clinical evaluation indicated the Farco-fill® inflation solution was only able to reduce encrustation in 34.5% of patients tested (Pannek and Vestweber 2011), though further clinical evaluation is required to fully understand the clinical efficacy of this product. A further possible issue with this approach is the potential for the emergence of resistant *P. mirabilis* strains, which have already been described in laboratory studies of *P. mirabilis* triclosan resistance (Stickler and Jones 2008). The mutants generated in these studies included those with high level triclosan resistance unaffected by inflation of retention balloons with triclosan solutions (Stickler and Jones 2008).

Urease inhibitors.

Owing to its critical role in the development of crystalline biofilms, the urease enzyme is a key target for control of catheter encrustation. Mutants of *P. mirabilis* lacking urease activity have been shown to be unable to form crystalline biofilms and block catheters, and a role for this enzyme in other aspects of *P. mirabilis* pathogenesis as also been described, including persistence in the upper urinary tract and renal damage during pyelonephritis (Jones *et al.* 1990; Johnson *et al.* 1993b; Dattelbaum 2003; Schaffer *et al.* 2016). Although numerous potential urease inhibitors have been identified from a wide range of sources, many have failed to progress beyond laboratory studies due to issues of toxicity or stability, and few

compounds are available for clinical use (Hassan and Šudomová 2017; Kafarski and Talma 2018).

In terms of urinary tract infection and catheter encrustation, acetohydroxamic acid (Lithostat®) has been shown to have efficacy in the reduction of bladder stone formation, catheter encrustation, and the resolution of chronic infection by urease producers when used in synergy with antibiotics (Griffith *et al.* 1979; Burns and Guthhier 1984; Griffith *et al.* 1988; Griffith *et al.* 1991). However, the use of this drug is undermined by a range of side effects and complications related to toxicity, and is often poorly tolerated by patients. Currently lithostat is only recommended for use when other treatments have failed and in specific groups of patients. Nevertheless, the clinical application of lithostat continues to benefit many individuals with chronic urinary tract infection by urease producers, and has confirmed the inhibition of urease as a viable approach to control catheter blockage. Further development of this approach may include the desgin of localised delivery systems that negate the need for systemic administration of drugs such as lithostat, which could avoid side effects and expand the range of patients who may benefit from these treatments.

Early warning systems.

The detection and signalling of events that indicate blockage of catheters may be imminent have also been explored as a strategy to help control catheter blockage, and reduce the complications associated with crystalline biofilm formation. The basic principle is to alert patients or carers that a catheter is at risk of blockage so intervention can be provided before complications arise. Early warning systems described so far have focused on the detection of pH changes in urine indicative of infection with *P. mirabilis* and other urease producers, and a key parameter in the formation of crystalline biofilms (Stickler *et al*. 2006a; Stickler *et al.* 2006b; Long *et al*. 2014; Milo *et al*. 2016; Zhou *et al*. 2018; Milo *et al.* 2018).

The first early warning system was described by Stickler *et al.* (Stickler *et al*. 2006b) and consists of a cellulose acetate matrix incorporating the pH indicator Bromothymol Blue, which changes from yellow to blue over the pH range 6-8 (Stickler *et al*. 2006b). These sensors were evaluated initially in *in vitro* bladder models, positioned either in the drainage bag or within tubing between the junction of the catheter and drainage bag connection, and provided up to 43 h advanced warning of blockage (Stickler *et al*. 2006b). Although highly promising in laboratory studies, clinical evaluation of this pH based chemical sensor approach has indicated that sensors may be activated weeks before blockage occurs, and identified considerable patient to patient variation in sensor performance (Stickler *et al*. 2006a; Long *et al.* 2014). These studies suggest that while these pH sensor based early warning systems are potentially useful for many patients, improvements are required in the accuracy and consistency of blockage prediction, as well as a reduction in the duration between sensor activation and blockage.

A variation on this approach has also recently reported by Milo and colleagues who developed pH responsive drainage bag sensors and coatings for catheters (Milo *et al*. 2016; Milo *et al*. 2018). These were based on incorporation of the dye carboxyfluorescein within a hydrogel matrix encapsulated by the pH responsive Eudragit polymer (Milo *et al*. 2016; Milo *et al*. 2018). In this system dissolution of the Eudragit polymer at elevated pH allows diffusion of the carboxyfluorescein indicator out of the hydrogel layer and into the urine, where it imparts a distinctive bright green coloration that signals the potential for blockage (Milo *et al*. 2016; Milo *et al*. 2018). In laboratory models this pH sensor technology provided 12-14 h advance warning of blockage, raising the possibility that this may provide a more accurate prediction of blockage, with shorter times between sensor activation and blockage (Milo *et al.* 2016; Milo *et al.* 2018). Furthermore, the design of these sensors mean therapeutic agents may also be loaded into the hydrogel matrix to provide “theranostic” systems capable of providing both early warning and directly combatting infection when

release is triggered (Milo *et al*. 2017; Zhou *et al.* 2018). However, this technology has not yet been subject to clinical evaluation and so it is currently uncertain how well this sensor technology, or any theranostic derivative, will perform under real-world conditions.

Bacteriophage.

The potential for bacteriophage (or phage) to provide an effective means of biofilm control in the context of CAUTI have been explored by several groups, and encouraging results reported (Curtin and Donlan 2006; Carson *et al*. 2010; Fu *et al.* 2010; Lehman *et al.* 2015; Melo *et al*. 2016; Nzakizwanayo *et al.* 2016; Milo *et al*. 2017). The properties of these bacterial viruses are particularly relevant to the eradication of biofilms, and aside from their ability to infect and kill bacterial cells, phage often possess attributes such as polysaccharide depolymerases that enable them to penetrate the bacterial biofilm matrix and infect constituent cells (Sutherland *et al*. 2004; Lu and Collins 2007). This feature of phage has been shown to facilitate the disaggregation and dispersal of biofilms (Sutherland *et al.* 2004; Lu and Collins 2007).

Most recently, bacteriophage therapy has been shown to reduce *P. mirabilis* crystalline biofilm formation on catheters, and to prevent catheter blockage (Nzakizwanayo *et al.* 2016; Milo *et al.* 2017). Using *in vitro* bladder models, Nzakizwanayo *et al.* (2016) showed that a single dose of phage significantly extended time to blockage under conditions simulating established infection with *P. mirabilis*. When the same dose of phage was applied to models simulating early-stage infections, the treatment eradicated the infection and catheters continued to drain freely without encrustation for a period of 8 days until experiments were terminated (Nzakizwanayo *et al*. 2016).

Further work has also demonstrated the potential to incorporate phage into infection responsive coatings triggered by urine pH elevation, providing the potential for automatic release of phage into the urinary tract to combat crystalline biofilm formation during the early stages of infection (Milo *et al.* 2017). More complex multispecies catheter biofilms have also been shown to be susceptible to phage therapy, with two-species biofilms of *P. mirabilis* and *Ps. aeruginosa* reduced by up to 99.9% in continuous flow models over a period of 48 hours using polyvalent phage cocktails (Lehman and Donlan 2015). The potential to formulate mixtures of phage capable of eradicating target organisms is also considered an advantage of this approach in terms of offsetting the development of resistance.

However, despite a renewed interest in phage therapy in light of increasing levels of antibiotic resistance, and mounting evidence for the efficacy of these viruses in treatment of bacterial infection, the development of phage therapy products intended for clinical use remains challenging. Particular challenges arise from the often narrow and strain specific host range of phage (which complicates development of broadly applicable phage mixtures), as well as issues related to production of phage preparations of the quality required for medicinal use, their route of administration and delivery, and the lack of a clear regulatory framework for phage products (Pirnay *et al.* 2015).

Efflux pump inhibitors.

Recent studies highlighting the role of efflux systems in bacterial biofilm formation, including *P. mirabilis* crystalline biofilm formation and catheter blockage, have led to the evaluation of compounds that inhibit these molecular pumps (efflux pump inhibitors; EPIs) for control of catheter biofilms (Kvist *et al.* 2008; Amaral *et al.* 2010; Holling *et al*. 2014b; Nzakizwanayo *et al*. 2017). Of particular significance are findings that drugs already used in human medicine can function as EPIs and attenuate the formation *P. mirabilis* crystalline biofilm formation

(Nzakizwanayo *et al*. 2017). Drugs from classes including the selective serotonin reuptake inhibitors and phenothiazines were found to exhibit putative EPI activity, with fluoxetine and thioridazine subsequently shown to significantly reduce crystalline biofilm formation and delay catheter blockage in *in vitro* models (Nzakizwanayo *et al.* 2017; **Figure 4**). This not only points to efflux inhibition as a viable target for control of *P. mirabilis* crystalline biofilm formation, but also the potential to repurpose a range of already licensed drugs to control these infections.

Summary

Bacterial biofilms remain a major problem in the care of many patients and continue to undermine the successful treatment of many infections. Patients managed by long-term urethral catheterisation are particularly vulnerable to biofilm related infections, with crystalline biofilm formation frequently leading to serious clinical episodes, emergency hospital referrals, and chronic long-term complications. Despite the large numbers of patients who are affected, and the enormous financial and human cost of these infections, there remains a general lack of awareness regarding the scale and impact of this problem (Prinjha and Chapple 2013). As the population of older individuals in many countries continues to rise, so too will the population of individuals subject to long-term catheterisation, along with the incidence of morbidity and mortality arising from associated infections and complications like crystalline biofilm formation.

Although notable progress has been made by dedicated researchers and healthcare practitioners in a number of areas (which has undoubtedly benefitted many patients), truly effective and widely applicable strategies to control biofilm related complications faced by

catheterised patients remain elusive. As Feneley and colleagues have recently pointed out (Feneley *et al.* 2015), finding a solution to this important clinical problem should be achievable, but will require an interdisciplinary effort from the scientific community, as well as greater engagement and support from research funders, regulators, and industry.

Figure legends:

Figure 1: The Foley catheter**. A)** Example shows a standard 14Ch Bard all silicone catheter with retention balloon inflated. **B)** Shows enlarged view of catheter tip and balloon region. **C)** Cross-section through catheter showing inflation line and main drainage lumen.

Figure 2: Proteus mirabilis crystalline biofilms**.** Examples of *P. mirabilis* crystalline biofilms formed on standard all-silicone Foley catheters, in *in vitro* models of the catheterised urinary tract. **A)** Scanning Electron Microscope image of catheter lumen showing formation of crystalline biofilm. **B)** Environmental Scanning Electron Microscope (ESEM) image of longitudinal cross-section of catheter lumen showing crystalline biofilm. **C,D)** Higher magnification ESEM images of established crystalline biofilms showing embedded struvite crystals. Images were generated by Dr N Holling. Part A is reproduced from Holling *et al.* 2014b, and part C is reproduced from Holling *et al.* 2014a.

Figure 3: Swarming in Proteus mirabilis. **A)** *P. mirabilis* swarming over an agar plates, demonstrating terraced appearance due to successive swarm cycles **B,C)** Scanning Electron Microscopy images of *P. mirabilis* swarmer cells fixed *in situ* during swarming over agar (Jones *et al.* 2004). Images demonstrated alignment and organisation of cells, as well

as interweaving of flagella filaments into helical connections between adjacent cells in the swarm front. Part C is reproduced from Jones *et al.* 2014.

Figure 4: Impact of repurposed drugs on crystalline biofilm formation (from Nzakizwanayo et al 2017). Images show impact of the repurposed efflux inhibitors thioridazine and fluoxetine on *P. mirabilis* crystalline biofilm formation in an *in vitro* bladder model. Models were run for 10h using standard 14 Ch all-silicone catheters. In treated models artificial urine media was supplemented with either thioridazine (400 µg/ml) or fluoxetine (128 µg/ml). Cross-sections of catheters directly below the eye-hole were imaged using scanning electron microscopy to visualise crystalline biofilm formation. Models run under these conditions until catheter blockage also showed models supplemented with either thioridazine or fluoxetine took significantly longer to block than untreated controls. Reproduced from Nzakizwanayo *et al.* 2017 (http://creativecommons.org/licenses/by/4.0/).

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Conflict of Interests

The authors have no conflicts of interest to declare.

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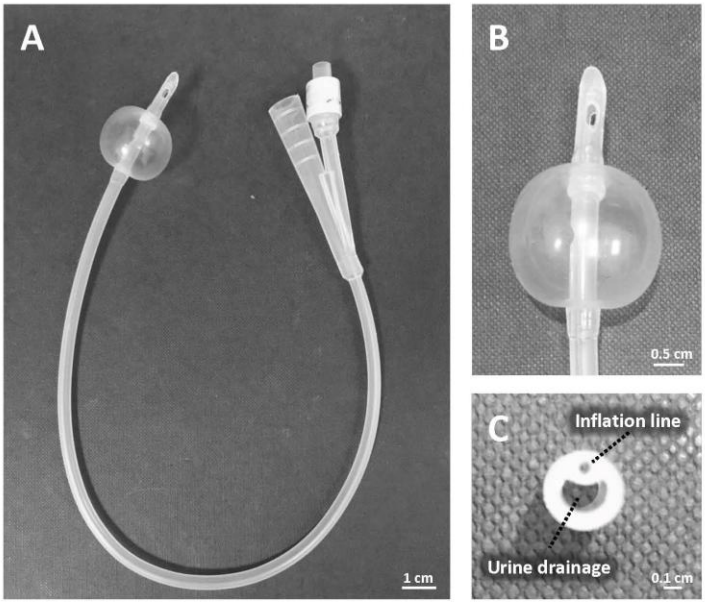

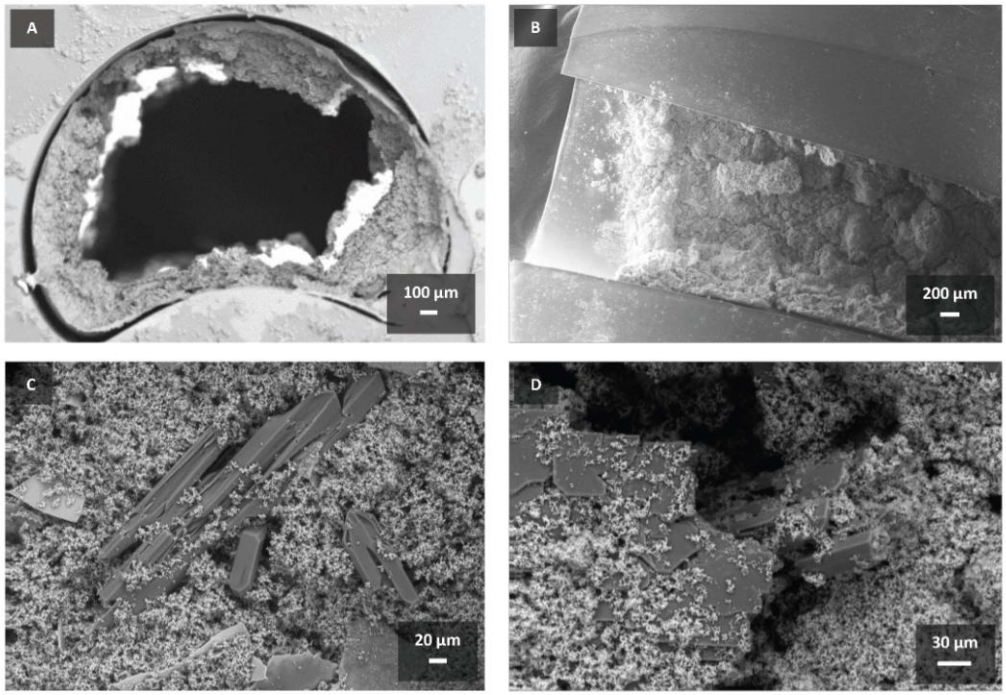

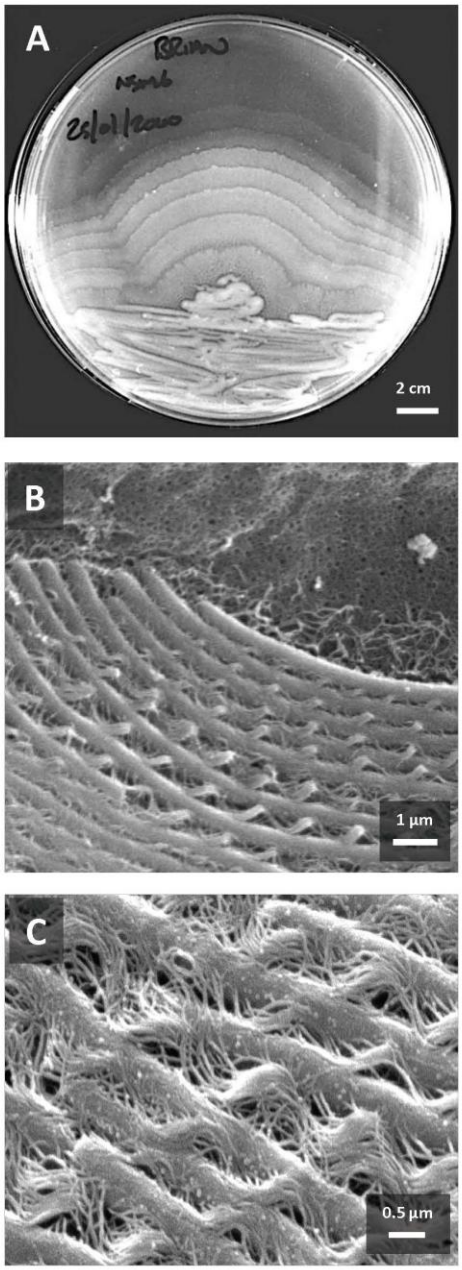

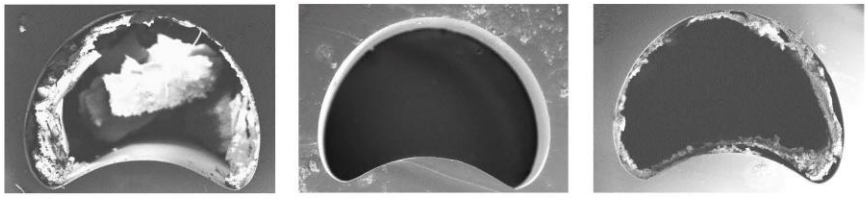

Untreated *+ Thioridazine* *+ Fluoxetine*